Exploring Microbially-Driven Methane Oxidation in Aerobic and Anaerobic Sediments

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Background

Methane is cited as an important greenhouse gas, with a heat-trapping potency approximately 21 times greater than carbon dioxide (Wuebbles and Hayhoe 2002). Landfills, livestock, and rice cultivation represent major potential inputs of methane to the earth’s atmosphere; however, it is estimated that only a small fraction of the subsurface methane pool is released. Aerobic methane oxidizing bacteria (methanotrophs) are capable of scrubbing large quantities of methane at the oxic/anoxic boundary, thus preventing transfer to the atmosphere. However, sulfate and nitrate may act as terminal electron acceptors for methanotropic archaean/bacterial communities, thus representing an anaerobic “scrubbing” mechanism (Girguis, Cozen et al. 2005; Raghoebarsing, Pol et al. 2006). Interestingly, no organisms (to date) have been shown to couple the oxidation of methane to iron (hydr)oxide reduction, despite the widespread distribution of these common minerals. Thus, the goal of this proposal is to isolate a microorganism or consortia capable of coupling methane oxidation to iron(III) reduction, and to examine the rates of methane oxidation by aerobic and anaerobic methane oxidizing bacteria.

Rational

Oxidation of methane coupled to iron(III) reduction (in this case, ferrihydrite—Fe(OH)$_3$) is a favorable, base producing reaction:

$$4\text{Fe(OH)}_3 + CH_4 \rightarrow 4\text{Fe(II)} (aq) + HCO_3^- + 6\text{H}_2\text{O} + 3OH^-$$

$$\Delta G = -2471 \text{ kJ/mol}$$

Ferrihydrite is a widespread mineral found within soils and sediments throughout the world (Cornell 2003). The occurrence of buried or partially buried organic carbon, from either anthropogenic (landfills) or natural (floodplain) activity, represents locations where methanogenesis may occur within close proximity to oxidized environments. Thus it is feasible that methane may diffuse into oxidized, iron(III) rich zones where methane oxidation may be couple to iron(III) reduction (Fig 1).

Although an iron-reducing, methane oxidizing bacteria has yet to be isolated, numerous groups have isolated aerobic, methane oxidizing bacteria. These organisms should be easy to culture, given a minimal salts enrichment containing methane and bicarbonate as the sole carbon pool.

Figure 1. Environment Conducive to Iron (hydr)oxide-methane close contact. Buried organic carbon (e.g. trees) and reduced sediments underlie, and are in close contact with oxidized, Fe-rich sediments.
Methods

General Enrichment Strategy

Two enrichment strategies will be utilized to select for aerobic and anaerobic methane oxidizing bacteria. More specifically, oxygen and ferrihydrite (amorphous iron hydroxide) will be provided for aerobic and anaerobic cultures, respectively. The source of inoculums consists of sediments, iron mat material, and ferrihydrite coated sand that was pre-planted for 1 week within the marsh sediments. The ferrihydrite coated sand inoculum was placed within swamp sediments in an effort to "bait" methane oxidizing, iron reducing bacteria. All samples were cored or packed tightly into containers to maintain anaerobiosis (when necessary, i.e. using the inoculum for anaerobic enrichments). Inoculum used in this study was gathered from the School Street marsh in Woods Hole, MA (Fig 2). The marsh material was immediately transported to the lab, and inoculated into sterile anaerobic or aerobic media. Hungate technique was utilized to ensure anaerobiosis in ferrihydrite-methane enrichments, and aerobic cultures were inoculated in air. Aerobic vials were crimp-sealed in air, and anaerobic serum vials (120 or 160 mils) were then crimp sealed and gassed with (80:20%) N₂:CO₂. Both sample types were then charged with 0.5 atm (~7psi) of pure methane. Natural gas was not used to avoid the biologically-driven transformation of ethane to formaldehyde, which could potentially poison the cultures.

Anaerobic cultures exhibited little sign of growth over a 2 ½ week period, and were thus not transferred to a secondary enrichment, save one culture which showed some promise of growth, as evidenced by darkening. However, aerobic cultures were generally turbid after 2-3 days, and were thus transferred multiple times.

Aerobic Methane Oxidation Experiments

Several aerobic cultures exhibited rapid growth in the presence of 0.5 atm methane; therefore, the rates of methane oxidation by these cultures were more closely examined. Approximately 108 cells of tertiary enrichment were spiked into fresh, sterile media charged with 2, 0.5, 0.05, and 0.005 mmoles of methane. Methane consumption (Fig 3), CO₂ generation, and turbidity were then measured as a function of time. Machaelis-Menton kinetics was used to compare the relative rates of methane oxidation by each culture. This was evidenced by extensive bubbling upon sediment perturbation.

Figure 2. Iron-rich surface materials (mat) near an Fe(II)-rich groundwater seep. Methane was present within the underlying sediments as evidenced by extensive bubbling upon sediment perturbation.
achieved by determining an initial rate of methane oxidation by each culture at each methane concentration (Fig 3), and then plotting the reciprocal initial rate vs. the reciprocal substrate concentration to yield a Lineweaver-Burke plot (Fig 4). The y-intercept of this plot is known as the Km, or substrate concentration at half-maximum, whereas the x-intercept is known as the maximum velocity (in enzyme kinetics, the point at which all enzyme is bound to with substrate).

**Microscopy**

Live/Dead staining coupled with fluorescence microscopy was used to visualize cells in aerobic, methane reducing cultures. Syto 9 is a dye which generally penetrates the membranes of both viable and non-viable cells, whereas propidium iodide, the second dye used in the stain, penetrates the membranes of damaged cells only. Thus the stain gives a good, approximate indication of cell viability. Figure 5 represents an image collected 7-21-06 from an aerobic methane oxidizing enrichment from School Street Marsh.
Isolation of Methane-Oxidizing Bacteria

Aerobic, methane oxidizing bacteria were obtained from enrichments by plating onto a minimal agar media, and storing the plates in a sealed jar containing 1.0:0.5 atm air:methane. After several days, both small and large colonies with varying morphology were observed. They were picked with a sterile toothpick, placed into PCR mastermix, and amplified with 8F-1492R (16S) primers for sequencing and subsequent identification. The pmoA gene (see below) was also amplified in isolates in an effort to better identify them as methane oxidizing bacteria.

Functional Gene Libraries and Restriction Digests

Functional pmoA gene libraries were constructed by using DNA-specific primers (A189, A661, A662) for this particulate methane monooxygenase subunit (Costello and Lidstrom 1999; Knief, Lipski et al. 2003). The amplification products were then ligaged into a TOPO R cloning vector and transformed into E. Coli, which was then grown on LB agar; this particular vector possesses a suicide gene, which if not interrupted by a ligation event, will kill the cells. Any colony could therefore be picked with a sterile pipette tip and grown for further plasmid prep and sequencing. The pmoA gene libraries were checked for vector contamination and chimeras, and then checked for homology against the NCIB database of known gene fragments. Our clones and their most closely aligned sequences were then imported into ARB, aligned with ClustalW, and placed in a phylogenetic tree using Phylip and 1000 bootstrap iterations. The same procedure was used to construct a 16S phylogenetic tree from 16S sequences collected from isolated bacteria, although alignment was achieved within ARB utilizing the greengenes database to find most closely homologous bacteria.

To further examine the diversity of pmoA genes within original soil and enrichment samples, restriction digests were performed on amplified pmoA DNA products. MseI (cuts within TTAA sequences at T_TAA) and MspI (cuts within CCGG sequences at C_CGG) were spiked in pcr product at a loading of 1000 units per enzyme. The digest was then incubated at 37C for 2 hours, after which amplification products were visualized on a Metaphor 2.5% agarose gel (Fig 6)

Figure 6. Agarose gel electrophoresis of pmoa restriction digest.
Bagasse Project

Another group of students in the 2006 MBL class is trying to utilize begasse, a common, cellulose-based fibrous sugar cane by-product, in fermenters to generate methane, organic acids and sludge. Resulting proteins and nutritious by-products could be then used to feed fish in developing countries (e.g. Haiti), and thus generate a sorely needed source of protein for impoverished people. Thus, a small methane-oxidizing bioreactor was constructed to determine whether appreciable biomass could be generated from methane oxidizing enrichment cultures. A 3L bottle (Fig 7) was filled with minimal salts media (freshwater) and 3 mM bicarbonate. Methane was continuously bubbled through the media at a rate of approximately 60 mL min$^{-1}$, and turbidity was measured as a function of time.

![3L Jar containing minimal salts media (Freshwater) and 20 mL of inoculum from methane-oxidizing enrichments. (Left) Reactor at time 0. (Right) Reactor after 7 days. Note high degree of turbidity and biofilm forming on walls of reactor.]

Results and Discussion

Anaerobic methane enrichments

The overall stoichiometry of methane oxidation via the reduction of ferric iron suggests that 4 moles of Fe(II) should be generated per mole of methane. However, in the enrichments which exhibited the most iron reduction, less than one-third Fe(II) was produced relative to the
overall decrease in methane concentration over the course of the enrichments (Fig 8).

![Graph showing Fe(II) produced and Methane Loss over time for different samples.](image)

Figure 8. Change in methane concentration over a 2 1/2 incubation period (light orange bars) and increase in Fe(II) concentration over the same period.

Additionally, methane concentrations within control vials (abiotic) decreased, indicating methane was likely escaping from the system. Unfortunately, we cannot ascertain the presence of methane oxidizing, iron reducing bacteria from these results. Iron reduction within some of the enrichments could be an artifact of the original inoculum, which likely contained some organic carbon, as well as iron reducers. The doubling time of nitrate reducing, methane oxidizing bacterial/archaeal communities is quite high (>14 days); if similar organisms reduce iron, it may take months, perhaps years to enrich for such organisms in this system.

Interestingly, the presence of Fe(II), either from initial inoculum or from microbial activity after inoculation, resulted in mineralogical transformation of ferrihydrite to other iron (hydr)oxides, including goethite, magnetite and hematite (as noted by distinct color changes, Fig 8). These transformations are clear indicators of microbial activity; thus, transformations occurring within future transfers will be possible indicators of methane oxidation (Fig 9).

Aerobic enrichments yielded multiple methane-oxidizing mixed cultures (Fig 10). Methane clearly decreased faster in aerobic enrichments than in abiotic controls (Fig 11), and CO₂ markedly increased in these enrichments, indicative of active respiration. Transfer cultures showed signs of increased turbidity after 1 ½ days, and were very turbid after 2-3 days.
Figure 9. Anaerobic iron (hydr)oxide enrichments. Color changes indicate biologically-induced transformation via Fe(II) production. Lighter coloration is likely a mix of ferrihydrite, hematite and goethite, whereas darker colors are indicative of magnetite.

Figure 10. Primary enrichment transfers after 1 day. Turbidity continued to increased for 2 days.

Figure 11. Decrease in methane (dark orange bars) and increase in CO2 (light orange bars) within aerobic cultures over 5 days of incubation.
**Rate Experiments**

Lineweaver-Burke plots constructed from rate experiments of 3 mixed cultures reveal a large disparity between the substrate concentrations at half-maximum methane oxidation rates (Km), as well as the maximum theoretical rate of oxidation (Vmax). Interestingly, these mixed cultures were grown from similar inoculum gathered in the same locality. Enrichments based on pond material gathered 30 cm below the subsurface yielded the highest rate of oxidation at ½ Vmax, whereas organisms enriched from a brown, iron-rich mat material also grew quite efficiently on methane; in contrast, methane oxidation rates in an enrichment derived from School Street March pond material were considerably slower, and the maximum velocity of methane oxidation from this enrichment was negligible compared to the other cultures. The deep pond material was anoxic, but may have been closer to the actual oxic/anoxic interface than the surface materials. Closer proximity to this boundary may explain the greater propensity for this enrichment to oxidize methane. Additionally, the mat material was in close proximity to an anoxic groundwater seep (in contact with oxygen), which may explain why this enrichment was adept at oxidizing methane relative to the pond’s surface material.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Km (mM)</th>
<th>Vmax (mM hr⁻¹)</th>
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<tbody>
<tr>
<td>Pond (30 cm)</td>
<td>0.23</td>
<td>3.7</td>
</tr>
<tr>
<td>Pond (surface)</td>
<td>4.7</td>
<td>0.0037</td>
</tr>
<tr>
<td>Road Iron Mat</td>
<td>0.65</td>
<td>9.5</td>
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**Isolation of methane oxidizing bacteria and phylogenetic analysis of pmoA**

Successful primary enrichments were plated on agar plates containing minimal media and 5 mM bicarbonate. After storage under a methane atmosphere for approximately 1 week, small and large colonies with varying morphology were identified. However, colonies often coalesced and were difficult to pick with a sterile toothpick, and also difficult to pick and re-streak. Nevertheless, multiple 16S sequences were obtained and placed within a phylogenetic tree along side organisms possessing the closest homology (Figure 12). It is clear from this tree that the isolates obtained in this study were quite novel, with only 2 organisms possessing >93% similarity to known organisms. These two organisms, 18_9M and 18_7 were most closely related to *Methylophilus sp.* and *Flectobacillus sp.*, respectively. Although *Methylophilus* is a known methane oxidizer, the ecological importance or niche of *Flectobacillus* is more nebulous. Other isolates obtained in the study did not possess a high degree of homology with other known organisms; they may represent yet-to-be described, novel organisms.
In addition to a 16S tree of known isolates, a tree was constructed from sequences obtained by amplifying pmoA from four enrichment cultures, two aerobic and two anaerobic. Three of the libraries, two aerobic and one anaerobic, did not form distinct clades; clones from these libraries were intermixed, and some of the clones possessed closest homology to known methane oxidizing bacteria, such as Methylobacter. However, most clones appeared to be novel, and many bore less than 90% resemblance to known pmoA genes. Furthermore, the clone library of an anaerobic enrichment containing ferrihydrite and methane formed its own clade (Fig 13). Thus, a novel suite of pmoA, amoA, or other genes may have been amplified. With the exception of the previously mentioned anaerobic enrichment, the remaining three treatments did not appear to follow any trends or cluster together.

Amplification of the pmoA gene also resulted in amplification amoA (ammonia monooxygenase subunit A) in a sample. After phylogenetic analysis, this clone bore close resemblance to the amoA gene found in Nitrosolobus multiformis, even though it formed a distinct clade which included known pmoA genes. This result illustrates the similarities between the amoA and pmoA genes, which are indeed closely related in structure and function (Holmes, Costello et al. 1995).

Finally, restriction digest of the pmoA amplification products revealed complex banding patterns in several samples (Fig 6). Although many enrichments possessed variable and complex banding patterns, starting materials (sediments) did not appear to possess a higher degree of complexity than enrichments, suggesting our enrichments were indeed selecting for methane oxidizing bacteria that were likely low in abundance in our original samples. Microscopy of enrichment samples revealed a suite of morphologies, and a complex biofilm formation that encapsulated a variety of living and dead cells (Fig 5).
Figure 13. Phylogenetic tree constructed from pmoA gene. Red and blue text represents pmoA from aerobic enrichments, whereas green text indicates pmoA amplified from anaerobic enrichments.

**Bagasse Project**

After several days, the methane oxidizing bioreactor’s turbidity had increased sharply, and continued to rise even as measurements were ceased (Fig 14). The rise in turbidity and biomass demonstrates that it may be possible to harvest biological material from a methane producing/methane oxidizing bioreactor for use in aquaculture.

Figure 14. Turbidity measurements from bagasse reactor as a function of time.
Concluding remarks

In this course, I attempted to prove that methane oxidation coupled to iron reduction could occur. Additionally, I measured rates of methane oxidation in aerobic methane-oxidizing enrichments, and used microscopy, chemical analysis, and molecular biology to characterize these samples. I found that anaerobic methane oxidation is most likely extremely slow, whereas aerobic methane oxidizers are very easy to culture and work with. We isolated organisms that are potentially very novel, and detected pmoA-like genes in our enrichments that differ considerably from known sequences.

Overall, the course has been an unforgettable and highly rewarding experience. It could not have been possible without the assistance of all of the lab teaching assistants, who were truly experts. The course directors, Tom Schmidt and Bill Metcalf were phenomenal lecturers and invited amazing guest speakers. Dion Antonopoulos tied the course together extremely well, and provided infrastructure that wouldn’t have otherwise been possible. Finally, Jared Leadbetter provided great lectures and useful discussion throughout the course.
References


